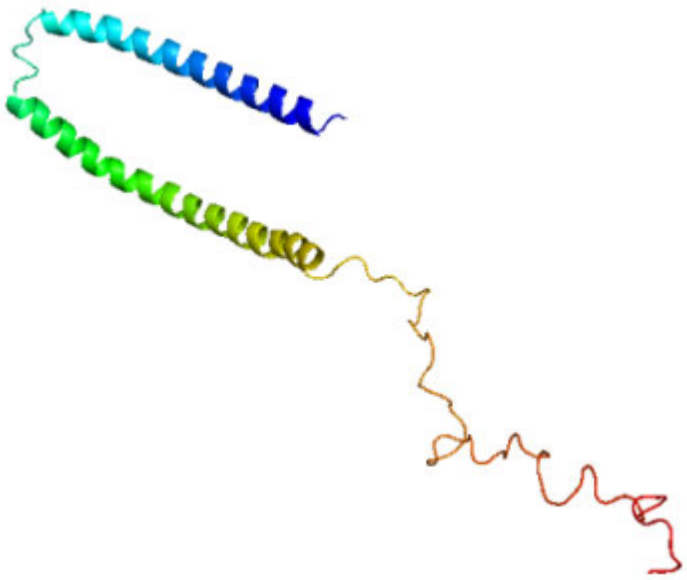
Drosophila and Parkinson's Disease:

The Effects of Various Stressors on Alpha-Synuclein Transgenic Flies



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Abstract

Parkinson's disease (PD) is a neurodegenerative disorder that reduces both quality and length of life. PD is characterized by a resting tremor, rigidity, and akinesia (impaired bodily movements); these and other symptoms are due to well-studied abnormalities such as the loss of dopaminergic (DA) neurons in the midbrain and the presence of inclusions, which in the case of PD, are aggregations of proteins and other substances in vulnerable cells. However, the underlying cause of these abnormalities is still a mystery.

A clue to the fundamental changes that lead to PD may lie in the intracellular inclusions, which are known as Lewy bodies and seem to be primarily composed of a protein called alpha-synuclein. Mutations in alpha-synuclein have been linked to several prominent cases of familial PD (an inherited form of the disease); since alpha-synuclein's function is unclear, further exploration may elucidate what role it plays in a healthy individual and how changes in the protein can lead to PD.

It is very difficult to directly examine what alpha-synuclein does in human subjects. Hundreds of PD patients and “healthy” human controls would be necessary for comparison and to reach statistically sound conclusions. There would be too many variables involved (e.g. nutrition and other environmental factors) that may obscure any connections that can be drawn between alpha-synuclein at the biochemical level and PD at the level of the human body as a whole. Therefore, many current experimental approaches involve simpler animal models. The fruit fly *Drosophila melanogaster* can serve as a fitting model for human PD, because introduction of the gene for alpha-synuclein leads to PD-like symptoms in the insect, including loss of DA neurons, reduced lifespan, and formation of alpha-synuclein-rich inclusions -all major characteristics of the human disease.

In my research, I used genetics and biochemical techniques to express (i.e. "turn on") the gene for alpha-synuclein in specific tissues and quantities in fruit flies to look for interesting phenomena and patterns. I also applied stress to some of these flies in two ways, either in the form of "wet" starvation (withdrawing food but not water) or by exposing them to paraquat, a chemical agent known to cause PD in humans.

Different combinations of the experimental factors (the presence/absence of the alpha-synuclein protein and its mutants, the type of stressor applied, and the sex of the fly) led to different longevities, or lengths of survival, for the flies. The results show that effectively modeling PD with alpha-synuclein transgenic flies requires expression in the right tissues, as well as the correct form of environmental stress (e.g. oxidation via paraquat).

Background

Parkinson's Disease

Ayurvedic text from ancient India, written in Sanskrit sometime between 4500 and 1000 B.C., contains the first known observations and effective treatment of the characteristic symptoms of Parkinson's disease (PD).[1,2] In terms of western history, Galen (A.D. 129 to ca. 199)-the Greek physician and writer-was perhaps the first to study the different types of tremors in limbs associated with PD in some depth.[3,4] However, the British surgeon James Parkinson was the first to describe several cases and distinct physical manifestations of the neurodegenerative disease in great qualitative detail in a 1817 monograph.[4,5] Thus, the disease now bears his name. Before then (and occasionally still found in modern literature), PD and parkinsonism was known vaguely in western medicine as the shaking palsy, or *paralysis agitans*.[4] In his classic treatise, Parkinson wisely suggested

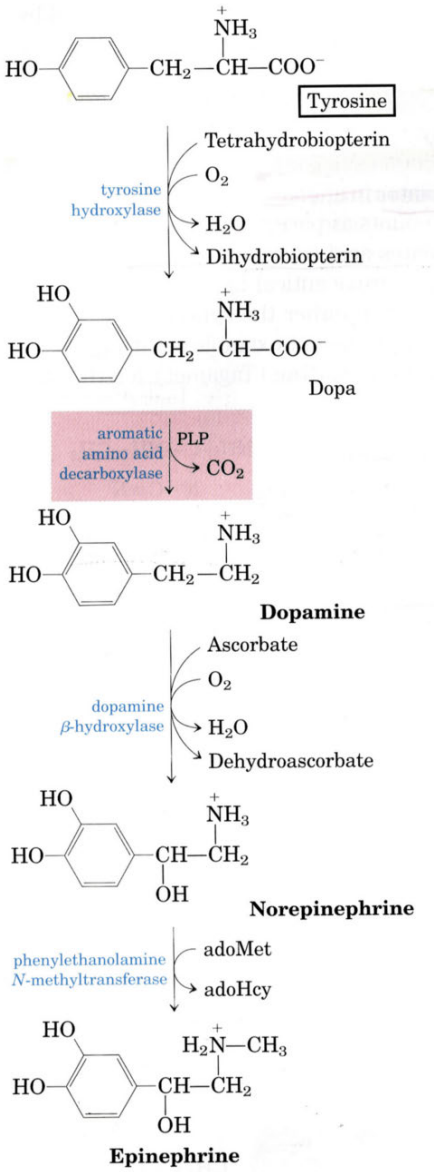
> Until we are better informed respecting the nature of this disease the employment of

> internal medicines is scarcely warrantable; unless analogy should point out some remedy the trial of which rational hope might authorize.[4]

Over 190 years later, Parkinson's disease continues to confound us. PD will afflict about 2% of people in their lifetime.[5,6] Aside from Alzheimer's disease, PD is the most common neurodegenerative disorder.[7] In 1998, over 1 million North Americans suffered from PD, and since age is the primary risk factor, this number will almost certainly increase with a large portion of the general population approaching 65 years of age, and PD is expected to affect 10% of people over 80.[1] Neurodegenerative diseases, including PD, "are projected to surpass cancer as the second most common cause of death among the elderly by the year 2040."[1] Furthermore, PD not only severely decreases quality of life, but also reduces life expectancy.[1]

Furthermore, a correct diagnosis is sometimes very elusive. Several major characteristics of PD are actually not unique to PD and may just be "parkinsonism" due to another cause (drugs, Wilson's disease, cortical-basal ganglionic degeneration, hemiparkinsonism-hemiatrophy, and/or one of over 20 other possibilities).[1] In fact, diagnoses of PD made prior to death are incorrect 24% of the time.[1] True PD can probably be most accurately diagnosed by observation of the three symptoms (a so-called "classic triad") of a resting tremor, rigidity, and akinesia, combined with asymmetry of symptoms and signs and a good response to levodopa; in the elderly, dementia occurs 6.6 times as frequently in elderly PD patients than as in elderly controls and thus often serves as another sign of PD.[1] There are two classifications of PD, sporadic and familial, which are quite similar, but familial PD cases generally manifest themselves at an earlier age and can be clinically atypical (i.e. feature deviations from the classical symptoms, patterns of progression, and so on).[8]

At the cellular level, Parkinson's disease causes characteristic, abnormal patterns of progressive cell death in a large variety of neurons, including the dopaminergic (DA) neurons of the pars compacta of the substantia nigra (for illustrations of this region of the midbrain and of its neurodegeneration in PD, see Figures 3a, 3b, and 3c), aminergic nuclei in the brain stem, hypothalamic neurons, small cortical neurons, the olfactory bulb, sympathetic ganglia, and parasympathetic neurons of the gut.[1] Of these, the loss of DA neurons in the substantia nigra seems the most documented and leads to local loss of dopamine, which, in turn, seems to cause akinesia and rigidity.[1, 9]



Lehninger Principle of Biochemistry, p. 860.

Figure 1. The biochemical pathway from tyrosine to dopamine and other catecholamines (epinephrine and norepinephrine). Pyridoxal phosphate is abbreviated as "PLP."[11]

As suggested above, since its discovery 40 years ago the compound levodopa remains the most effective and widely-used therapy for PD.[1] Levodopa, or L-dopa, is "the levorotatory form of dopa [a phenolic amino acid $C_9H_{11}NO_4$] that is obtained especially from broad beans or prepared synthetically."[10] In the brain, the amino acid tyrosine is the precursor for levodopa (see Figure 1, at right); the hydroxylation reaction that converts tyrosine to L-dopa is catalyzed by tyrosine hydroxylase and involves tetrahydrobiopterin as the reducing agent and oxygen as the oxidizing agent.[11] The levodopa can then be converted - via decarboxylation (catalyzed by a pyridoxal phosphate-containing enzyme called dopa decarboxylase, the "aromatic amino acid decarboxylase" in the figure) - to form the

neurotransmitter dopamine.[10, 11] Thus, administering levodopa or a dopamine agonist (i. e. a mimic) to a PD patient can somewhat counteract dopamine deficiency.[12] However, all medications for PD have adverse effects (for those of L-dopa, see Figure 2), and as mentioned earlier, the loss of DA neurons in the substantia nigra is just a small part of a much larger picture of PD. Furthermore, nothing has been proven to successfully slow the progression of PD.[12] New therapies that are currently explored and investigated for effectiveness include "neuroprotective" substances, deep brain stimulation[13], and surgical procedures, such as thalamotomy and pallidotomy.[1,12]

PROBLEMS AND COMPLICATIONS OF LEVODOPA THERAPY

PROBLEM	SYMPTOMS
Related to disease	
Early suboptimal symptom control	Varying response of symptoms to treatment
	Greater resistance of tremor than of other symptoms
Later treatment-resistant symptoms	
Motor	Dysarthria
	Freezing of gait (on-period freezing)
	Postural instability with falls
Nonmotor*	Dysautonomia, weight loss
	Sensory symptoms including pain (some may be responsive to levodopa)
	Changes in mood or behavior, sleep disturbances
	Cognitive dysfunction and dementia
Related to treatment and disease	
Motor fluctuations	Wearing off of drug effect (end-of-dose deterioration), concomitant fluctuations of nonmotor symptoms that may be as disabling as motor symptoms (or more so)[115]
	On–off phenomenon, more rapid and unpredictable fluctuations
Dyskinesias (abnormal involuntary movements)	Peak-dose dyskinesias; chorea, athetosis, and less often, more prolonged dystonia, often worse on initially affected side
	Diphasic dyskinesia ("beginning-of-dose" and "end-of-dose" dyskinesias), mixtures of choreoathetosis, ballism, dystonia, alternating movements (especially in legs)
	Off-period dystonia, most often involving legs and feet (including morning foot dystonia)
Psychiatric disturbances	Vivid dreams and nightmares
	Rapid-eye-movement sleep behavior disorder (may develop before parkinsonism[116])
	Visual hallucinations with clear sensorium
	Hallucinations with confusion
	Mania, hypersexuality
	Paranoid psychosis

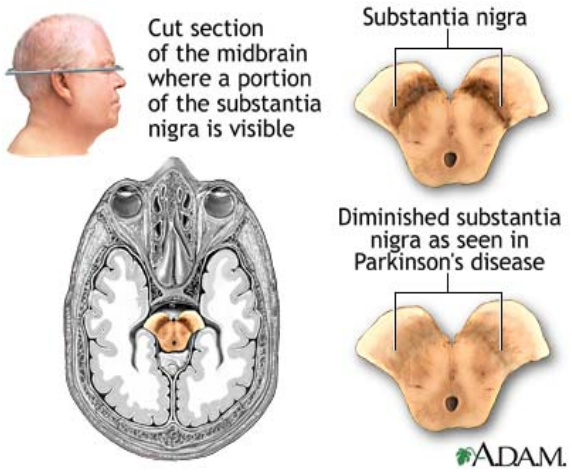
*These symptoms sometimes occur earlier in the course of the illness.

Lang, A., Lozano A., 1998. Parkinson's Disease-Second of Two Parts. NEJM 339, p. 1135.[12]

Figure 2. Even levodopa, currently the most effective treatment for PD, is somewhat complicated and has many side

effects that must be countered, generally by other medications (e. g. to limit peripheral side effects, carbidopa, a commonly used dopa decarboxylase inhibitor).[12]

(3a)



Medline Plus Medical Encyclopedia

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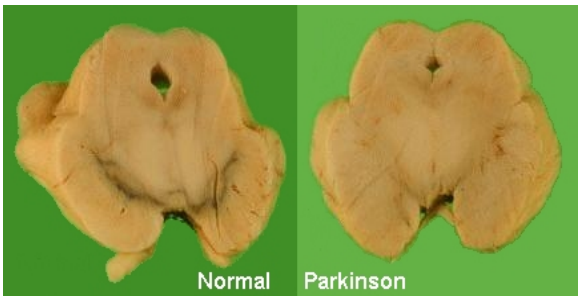

[*Anterior*]

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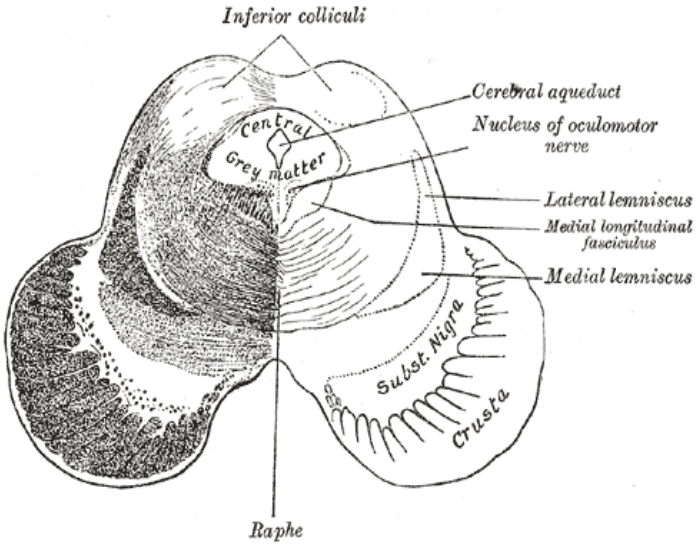
Figures 3a, 3b, and 3c. (Above left): The substantia nigra (Latin for "black substance")[14] is a region of the midbrain that can be clearly seen in a transverse section of the neural tissue[15], especially at the level of the inferior colliculi (below)[16]; normally, its dark coloration is due to the presence of DA neurons, which contain the pigment melanin.[17] In PD, the substantia nigra typically experiences a significant loss of DA neurons (above right)[18], which is visible upon dissection. "Approximately 60 per cent of nigral neurones have to be lost, with an 80 per cent depletion of striatal dopamine, before the symptoms of PD develop."[18]

(3c) "4b. The Mid-brain or Mesencephalon." Anatomy of the Human Body.

[*Posterior*]



[*Anterior*]

Some specific cases of Parkinson's disease appear to have a genetic/inheritable component, and PD in general may involve a combination of changes in gene expression and environmental factors. This and the other aforementioned fundamental beliefs have led to recent animal models for PD, such as *DJ-1* fruit flies. A few years ago, a *Drosophila* model for Parkinson's disease based on the gene *DJ-1* was developed by Meulener et. al.[19] In humans, *DJ-1* is a gene associated with sporadic (i.e. not inherited/familial) PD caused by exposure to certain environmental toxins, specifically those that cause oxidative stress (e. g. paraquat, rotenone, and hydrogen peroxide).[19] *Drosophila* have two main homologs, or genes that are similar due to common ancestry, to *DJ-1* in humans. These are known as *DJ-1α* (sometimes designated as CG6646) and *DJ-1β* (or CG1349), and both have protein products with at least 52% identity and approximately 70% similarity with the human protein.[19] Flies lacking any *DJ-1* function (double-knockout mutants missing both forms of *DJ-1*) are viable, fertile, not susceptible to pre-adult lethality, and develop without any significant external deviations when compared with wild-type flies (the control flies); mutant and control flies had similar lifespans.[19] However, when subjected to various toxins, a clear difference appeared. The double-knockout mutants were about 10-16 times more sensitive - measured by the percent of flies alive after a particular number of hours of exposure - to paraquat and hydrogen peroxide (H_2O_2), two chemicals that are powerful oxidants, than normal flies; DTT (dithiothreitol) and β-mercaptoethanol (BME), toxins that act through non-oxidative mechanisms, affected both groups similarly.[19] Using Western immunoblotting, the Muelener team also found that *DJ-1β* was expressed more ubiquitously (in the fly brain and body) than *DJ-1α* (with expression limited mostly to the testes), and thus seemed to be responsible for most of the paraquat sensitivity and perhaps for the hydrogen peroxide response as well.[19] This conclusion was supported by exposing single knockout mutants (missing either *DJ-1α* or *DJ-1β*) to paraquat; the *DJ-1α* knockout flies showed no significant differences in

survival when compared to the control flies, whereas the *DJ-1β* knockouts had a survival curve resembling that of double-knockout *Drosophila*.[19]

Though *DJ-1* mutant flies may seem to be a very promising model for PD induced by exposure to hazardous substances (e. g. paraquat) in humans, they lack a key PD characteristic discussed earlier in this paper: the neurodegeneration of DA neurons.[19, 20] In fact, a growing body of evidence suggests that the protein product of *DJ-1* plays neither a preventative role in terms of oxidative stress nor a direct role overall. Instead, the DJ-1 protein seems to be activated fairly rapidly in a damaging, oxidative environment and acts as a molecular chaperone that prevents aggregation of another protein called alpha-synuclein; in the case of alpha-synuclein, self-aggregation is abnormal and may lead to the previously mentioned structures called Lewy bodies, which have been associated with Parkinson's disease and other neurodegenerative disorders (e.g. Alzheimer's disease).[1, 6, 20, 21] The neurodegenerative diseases associated with alpha-synuclein are commonly referred to as "synucleiopathies."[8]

Alpha-Synuclein

Another striking characteristic of Parkinson's disease is the formation of abnormal intracytoplasmic strucutures called Lewy bodies ("degenerating ubiquitin-positive neuronal processes" or inclusions that accumulate filaments and trap cytosolic proteins); in PD, Lewy body formation occurs with neurodegeneration and takes place in certain "selectively vulnerable neurons"[22] of the "substantia nigra, locus ceruleus, nucleus basalis, hypothalamus, cerebral cortex, cranial nerve motor nuclei, and the central and peripheral divisions of the autonomic nervous system."[1,5,23] Lewy bodies or Lewy neurites (affected extensions of the neuronal cell bodies), are not exclusively found in PD, as mentioned earlier; instead, their appearance seems to correlate with neurological stress (e. g. toxic proteins or substances) or dysfunction.[1] Fibrils of an interesting protein known as alpha-synuclein have been found to be a major, if not the primary,

component of Lewy bodies.[24, 25]

Alpha-, beta-, and gamma-synuclein are small, soluble proteins of the synuclein family, found primarily in neural tissue and in some tumors; "all synucleins have a highly conserved alpha-helical lipid-binding motif with similarity to the class-A2 lipid-binding domains of the exchangeable apolipoproteins [i.e. proteins that bind with lipids to form lipoproteins]."[26, 27] Synucleins are found only in vertebrates, though they share some conserved structural similarities with certain plant proteins.[26] In addition, only alpha- and beta-synuclein are found significantly in the brain, and primarily in pre-synaptic terminals.[26] Unlike alpha-synuclein though, beta-synuclein - differing by only a 12-amino acid segment within the middle of a key section of the primary sequence called the NAC region (discussed in more detail later)- does not aggregate and is not found in Lewy bodies.[22] Alpha-synuclein is typically found in the cytoplasm, nucleus, and/or membranes, but is generally membrane-bound in DA neurons.[8] Current data suggests that alpha-synuclein normally functions in "the regulation of membrane stability and/or turnover" (especially for membranes associated with dopamine release and transport), although this has not been confirmed.[8, 26]

The wild-type human gene for alpha-synuclein, found on chromosome 4q and designated as *Syn* in this paper, is also commonly known by a variety of other names, including SNCA, PD1, NACP, PARK1, PARK4, and etc.[1] Three missense mutations of the *Syn* gene lead to three respective mutated forms of alpha-synuclein protein- Ala53Thr (also called A53T), Glu46Lys (or E46K) and Ala30Pro (or A30P). [1,5,28] Though mutations in *Syn* rarely causes PD, either one of these point-mutated genes (and the resulting/associated mutated proteins) or extra copies (e.g. triplication) of the wild-type gene has been found in families with an abnormally high occurrence of PD.[5, 22] In a study of a large Italian family, Syn^{A53T} was found to have a penetrance (or the frequency of a mutant *Syn* genotype corresponding with the disease phenotype) of about 85%; the same mutation was found in three smaller Greek families (who seem unrelated, but may be

"very distantly" related at most), in conjunction with a history of PD.[1, 5] Similarly, the unusual presence of *Syn*^{A30P} was noticed "in a family of German origin" due to many cases of PD.[1, 5] The PD phenotype associated with the alpha-synuclein gene seems to be inherited in an autosomal dominant pattern.[5] In terms of correlative evidence in an animal model for human PD, the rat protein SYN1 is 95% similar to human alpha-synuclein and is normally expressed in the same regions (in rats) of the nervous system as the areas where Lewy bodies are found in PD patients.[5] For some insight as to how mutated alpha-synuclein may lead to Lewy body formation, a key aspect of the disease state, examination of the protein's structure is necessary.

The primary structure of alpha-synuclein can be divided into three regions (see Figure 4b): the amphipathic region (alpha-helical and located between residues 1-61), the hydrophobic NAC (stands for non-β-amyloid component, which is associated with plaques in Alzheimer's disease -plaques that contain mostly β amyloid; residues 61-95), and the acidic region (residues 95-140).[29] In the amphipathic region and well into the NAC region/domain, there are at most seven 11-amino acid "imperfect" repeat sequences, containing some variation of the subsequence "KTKEGV" (UniProt only mentions the first four contiguous repeats of the subsequence "[E/G/S]KTK[E/Q][G/Q]VXXXX")[8]; hence an alternative way to divide and label the primary sequence of alpha-synuclein is by a "repeat region" and a "tail region."[30] A "chaperone-mediated autophagy recognition motif" is present from residues V95 to N99.[30] The NAC domain is necessary for aggregation/fibril formation in synucleiopathies, and forms the core of fibrils, such as those in Lewy bodies.[8, 22, ,29]

According to a "minimized average structure" based on 20 conformers (see Figure 5) and derived using NMR (nuclear magnetic resonance) spectroscopy, micelle-bound alpha-synuclein (e.g. alpha-synuclein attached to a vesicle) has one main chain and no disulfide bonds within this Chain A.[31, 32] The overall tertiary structure looks something like an uneven horseshoe with some thread dangling from its longer side. A short, fairly rigid linker region connects two large anti-

parallel alpha helices.[30, 32] The shorter of the two helical regions is closer to the N-terminus, and the other is followed by a "short extended region" (residues G93 to K97) with a long, flexible carboxyl tail (D98 to A140).[30] There are four hydrogen-bonded turns (N-terminus to C-terminus: between residues 41 and 42, 120 and 122, 124 and 126, and 130 and 131, respectively).[32] The derived structure also shows only about 1% of the protein existing as beta sheet -two acidic and single-residue beta-bridges at D119 and E123 (see Figure 6); the rest of the 140-amino acid (molecular weight: 14460 Da) does not have a fixed secondary structure, especially the hydrophilic carboxyl tail.[30, 32, 33] Unprocessed, wild-type alpha-synuclein is "predominantly a random coil in aqueous solution"[30] but becomes mostly helical (59%) when associated with lipids.[30, 32] There is evidence that the C-terminal tail regulates aggregation of alpha-synuclein and may determine the diameter of alpha-synuclein fibrils.[8, 30] Additionally, Ulmer et. al. suggests that alpha-synuclein may switch between a mostly helical to an "uninterrupted helix" conformation -a conformation necessary for alpha-synuclein multimer formation- based on lipid contact and perhaps self-associate through the aforementioned imperfect repeats.[8, 30] However, this sort of aggregation is different from the potentially harmful formation of extensive alpha-synuclein beta sheets, which will be discussed in the next paragraph.

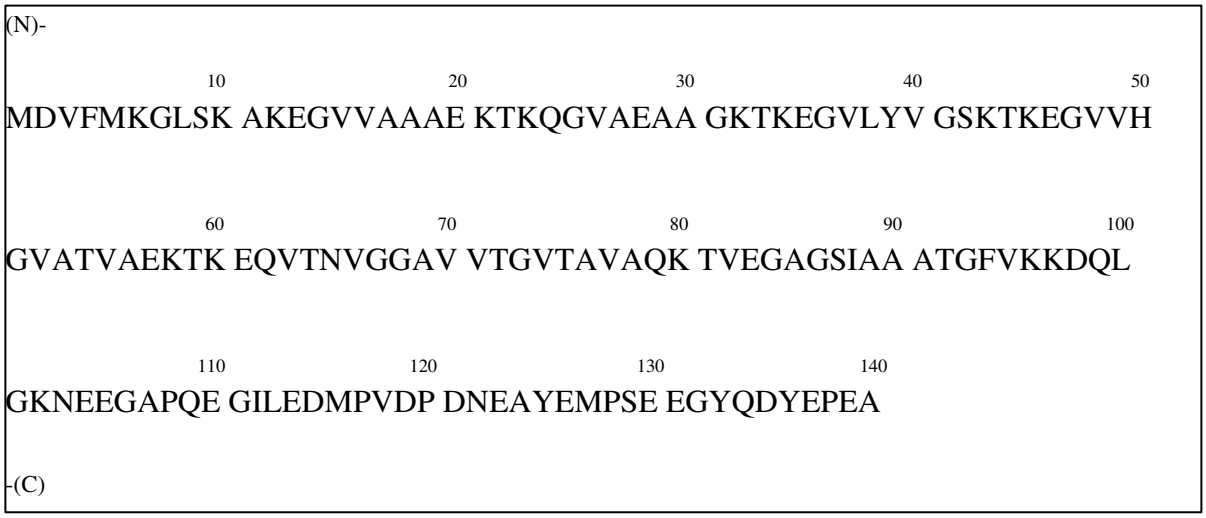


Figure 4a. (Primary sequence from UniProt; UniProt ID: P37840). The amino acid sequence of alpha-synuclein (PDB ID:

1XQ8; UniProt ID: P37840).[8, 31, 32] Alpha-synuclein's pI, or isoelectric point (i.e. the pH at which the protein has no net charge and a value useful for protein separation/purification techniques based on molecular charge), is estimated to be 4.47 ± 0.30 (a value that can vary depending on the pK_a values used in the calculation).[33, 34]

Amphipathic Region

MDVFMKGL (SKAKEGVVAAA EKTKQGVAEAA GKTKEGVLYVG SKTKEGVVHGV

NAC Region/Domain

ATVA EKTK|EQVTNVG GAVVTGVTAVA QKTVEGAGSIA)

Acidic Region

[AATGFV|KKDQLGKNEEGAPQEGILEDMPVDPDNEAYEMPSEEGYQDYEPEA]

Primary sequence from UniProt (UniProt ID: P37840)

Figure 4b. Another look at the primary sequence of alpha-synuclein. The vertical bars divide it into three sections (the amphipathic region, NAC or non-β-amyloid component region, and the acidic region)[22], while the parentheses and brackets mark the "repeat region" (by which "alpha-synuclein interacts with lipid membranes") and the "tail region."[31] A sans-serif font is used to emphasize tandem 11-amino acid imperfect repeats; the red residues (i.e. the first continuous set) are recognized by UniProt, whereas both the red and blue residues are proposed as repeat sequences by Ulmer et. al., who assigned coordinates (available in the RCSB PDB) to the residues of human micelle-bound alpha-synuclein using NMR spectroscopy.[30]

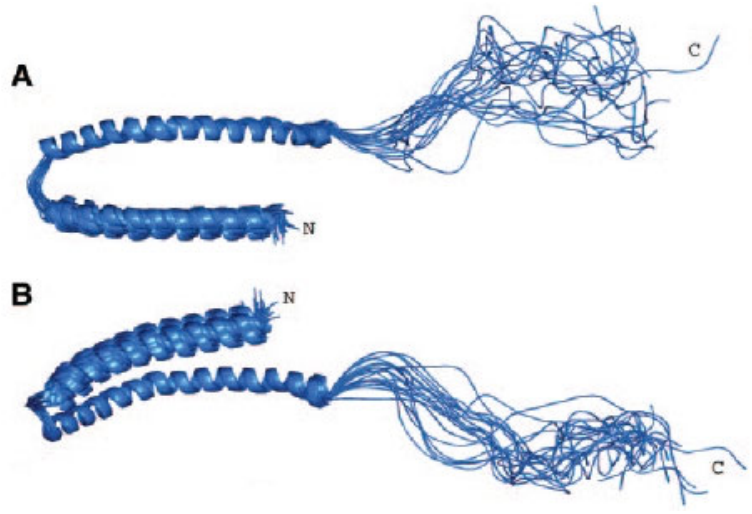


Figure 5. (from Ulmer et. al, 2005. Structure and Dynamics of Micelle-bound Human α-Synuclein. J. Biol. Chem. 280, p.

9599). Two different perspectives (A and B, which differ by approx. 120 degrees of rotation about the x-axis) of the twenty conformers of alpha-synuclein used to derive an average overall structure. The structure of conformers were determined using NMR spectroscopy and, in this image, are superimposed on the longer of the two alpha helices (residues K45-T92). Note the structured nature of the short linker between the helices and the highly flexible tail at the C-terminus (D98-A140).

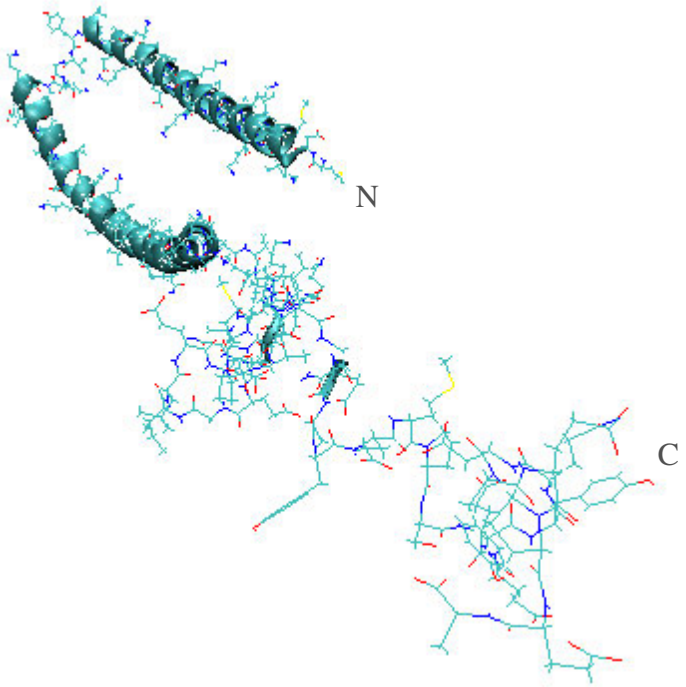


Image created using data from RCSB PDB (PDB ID: 1XQ8)[31] and VMD.[35]

Figure 6. A minimized average structure of wild-type human alpha-synuclein. This view of the protein is one with the tail of the C-terminus coming out towards the reader and to the right of the page. The important features of this line representation are the two alpha helices, as well as the two isolated, one-residue beta-bridges (thickened for visibility and near the center; D119 and E123), which are the beta strands/sheets in the protein. Cyan lines represent carbon atoms; red lines represent oxygen atoms; blue lines represent nitrogen atoms; and finally, the three yellow lines that are visible represent sulfur atoms (found only in methionine residues; no cysteines are present in alpha-synuclein).

As one of the three known genes (Syn^{A53T}, Syn^{A30P}, and Syn^{E46K}) for mutant alpha-synuclein protein, Syn^{A53T} serves as a good example of how a single point mutation can lead to

the disease state of PD. *Syn*^{A53T} differs from the wild-type gene at the fourth exon, where a single base pair change at position 209 (where a deoxyguanine becomes an deoxyadenine; also known as the G209A mutation) causes a change in the primary amino acid sequence during protein synthesis, an alanine-to-threonine substitution at position 53 (Ala53Thr; see Figure 7), hence the designation "A53T".[5]

> The Ala53Thr substitution is localized in a region of the [alpha-synuclein] protein whose secondary structure predicts an a helical formation, bounded by b sheets. Substitution of the alanine with threonine is predicted to disrupt the α helix [of wild-type alpha-synuclein] and extend the β sheet structure.[5]

β sheets, in turn, are thought to lead to the self-aggregation of proteins like as alpha-synuclein, forming plaques.[5] The A30P form of alpha-synuclein "behaves differently from the others" (i.e. the A53T mutation and overexpressed wild-type alpha-synuclein) and does not form large insoluble fibrils but "relatively soluble, partially aggregated species" instead.[28] How mutated alpha-synuclein leads to PD is not yet truly proven/known.[23] Perhaps due to some or all of these point mutations,

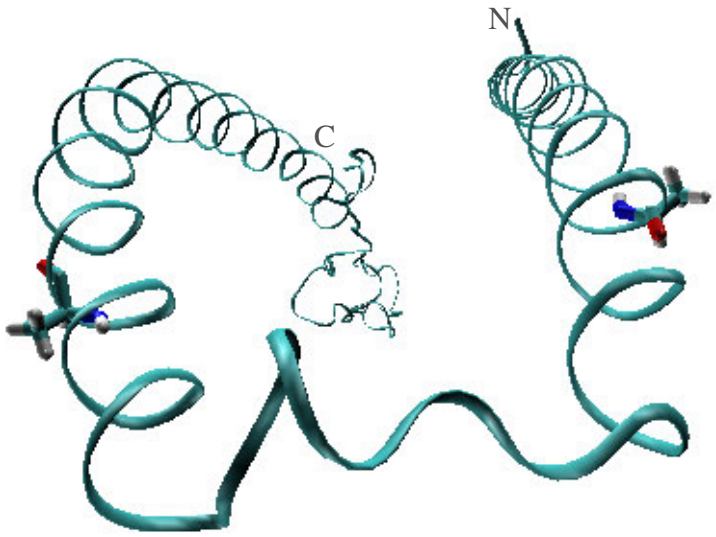


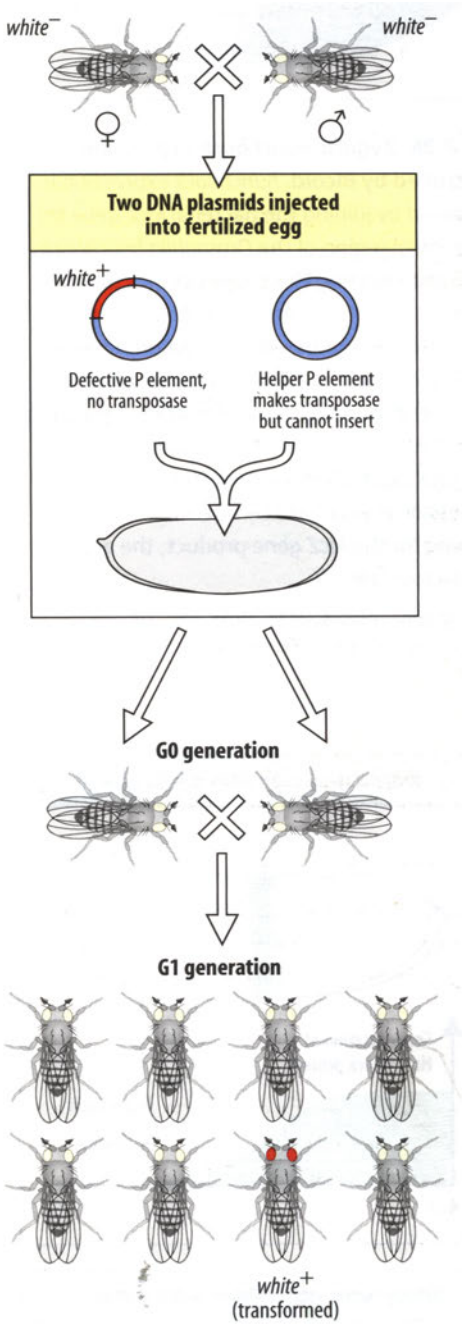
Image created using RCSB PDB (PDB ID: 1XQ8)[31], VMD.[35]

Figure 7. A ribbon representation of alpha-synuclein, facing the linker region (with the two alpha helices and both ends extending into the background) and with emphasis on the alanine residues at position 30 (A30; multi-colored structure at right) and position 53 (A53; similarly, at left). These are the two residues of interest, in terms of the α-*Syn*^{A30P} and α-*Syn*^{A53T} mutations (point mutations of wild-type α-*Syn*), respectively.

alpha-synuclein (normally "degraded by the proteasome [a protease that destroys misfolded proteins][36] in a ubiquitin-independent manner"), misfolds or is inappropriately modified, overwhelming the proteasome and leading to "aberrant ubiquitination and [Lewy body] inclusion formation."[37]

Transgenic Organisms

Trangenic organisms are organisms with genomes that contain genes from a different species via recombinant DNA methods.[11] For example, transgenic *Drosophila melanogaster* (containing a human gene or a gene from another organism) can be made using P-element-mediated transformation (outlined in Figure 8, at right).[38] This involves placing the gene in a transposon carrier - a P element - that has been isolated. P elements are short DNA sequences in *Drosophila* that can insert themselves into different places in a chromosome and "can also hop from one site to another within the germ cells."[38] An enzyme known as a transposase (made by the P element itself) catalyzes the transfer of the P element from one chromosome to the next.[38] In addition to the non-*Drosophila* transgene, a marker gene generally has to be added (e. g. *white*$^+$ to flies homozygous for the white-eye mutation, *white*$^-$); expression of this marker gene causes a



Wolpert, L. et. al. *Principles of Development.* 3rd Ed. (2007). p. 59.

Figure 8. An example of P-element-mediated transformation, a technique that can be used to create transgenic organisms.

certain phenotype (a physical manifestation) to appear, confirming a successful transformation.[38] Because hopping can lead to "genomic instability," the transposase gene of the carrier P element is removed; a helper P element that can synthesize transposase but cannot insert is co-injected with the carrier P element into a fertilized fruit fly egg, which will develop into an adult with any transformed cells confined to the germ cells. In the next generation (i. e. after crossing flies injected with the P elements), some of the offspring will have the carrier P element, with its transgene, in their somatic cells (e. g. for the *white*$^{+}$ marker introduced above, only red-eyed offspring will have been transformed).[38]

Transgenic mouse models (some with human alpha-synuclein protein) have been partially successful, in terms of representing PD. Mice overexpressing human *Syn* have "reduced motor performance," and those with *Syn*^{A53T}, instead of the wild-type gene, show "early and dramatic decline of motor function."[39] Though these transgenic mouse models provide some of the physical, locomotor-related manifestations of the human disease state, they do not display a significant loss of DA neurons in the substantia nigra.[39, 40] Furthermore, as my research mentor suggested in our initial discussion of the project, overexpression of alpha-synuclein in mice may lead to some DA cell loss that, in turn, causes the death of other cells indiscriminately; this may complicate the results being reported by other groups. This is why we chose an approach based on tissue-specific overexpression.

Experimental Methods

Fly Husbandry

The Drosophila larvae and adults were raised in a fly stock room (at 22 degrees Celsius), in small, transparent plastic tubes/vials with cornmeal-based food at their lower ends and either

cotton plugs or corks (to contain the adult flies); these food vials were labeled with the date when flies were first present and the genotype of the flies. The flies were exposed to light from the fluorescent lamps in the ceiling of the stock room almost every morning to keep their metabolic cycles fairly steady, and the lights were turned off at night. To prevent overcrowding due to the females laying an excessive number of eggs, which results in stunted growth (i.e. smaller larvae and adults), the adults were generally removed -often transferred to empty food vials to mate and increase the available stock- every few days. For instance, flies of the *C155Gal4* line were known to mate and lay eggs at a younger age than the other lines; these adults had to be removed every two or three days, as soon as the larvae began emerging. After 15 days, the food vials were disposed due to possible eclosure (i.e. emergence of an adult insect from its pupa case) of flies of the F2 (second filial) generation (from mating between the F1 adults), which may have undesirable genotypes.

Fly Strains and Genetics

Instead of using the simple P-element-mediated transformation system for *Drosophila melanogaster*, as described earlier, *Syn* (or SNCA) and alpha-synuclein mutant genes Syn^{A30P} and Syn^{A53T} were successfully overexpressed in a tissue-specific manner via a Gal4 driver/UAS system. Gal4 (or "Gal4p") is a protein naturally found in yeast, or more specifically a yeast "DNA-binding transcriptional activator,"[11] and plays an important role in turning on the transcription of certain genes needed to metabolize galactose when the sugar is present as a food source for the yeast.[11] Gal4 is also a useful tool for activating genes in a non-yeast organism, such as *Drosophila*. In a typical scheme involving targeted gene expression and Gal4 drivers, a "pattern line" is crossed with a "target line."[36, 38, 41] (This is illustrated and further explained in Figure 9). The Gal4 gene can be introduced into *Drosophila* to create "well-characterized lines

that express the yeast activator in a variety of tissue- and cell-type-specific patterns [i.e. the pattern lines]"[11] via the enhancer-trap technique.[38] In this technique, the Gal4-coding gene is placed in a vector (a P element) that can randomly integrate into the fly genome.[38] The gene for Gal4 then falls under the control of "the promoter and enhancer region adjacent to its site of integration, and so Gal4 protein will be produced where or when that gene is normally expressed."[38] Many Gal4 lines (i.e. strains) have been developed, and for these, the specific areas where Gal4 is expressed have been determined.

After the pattern lines are established/selected, the gene being investigated (e.g. *Syn*) is placed in a Gal4-sensitive expression vector (a plasmid), which is then introduced into wild-type flies, as in P-element-mediated transformation. Thus the relatively easily manipulated Gal4 expression can be used to drive (hence the term "driver") the expression of *Syn* in specific tissues and is very useful for exploration of the role of alpha-synuclein (and other proteins) at a cellular level.

According to Feany and Bender, alpha-synuclein transgenic flies (and flies with the mutant forms of the protein) seem to be useful and better than some other models, because they can replicate "three key features of the pathology of Parkinson's disease: adult onset, involvement restricted to the nervous system and anatomical specificity within the nervous system [particularly in two groups of DA neurons called the dorsomedial clusters]."[23] (In other words, the effects of introducing alpha-synuclein are limited to the nervous system and only particular, sensitive parts of the nervous system – just as in human PD). However, it should be noted that there is some significant controversy over this claim; overexpression of alpha-synuclein may not necessarily lead to the manifestation of PD-like features in *Drosophila*. Pesah et. al. undertook pan-neuronal, DA neuron-specifc, and eye-specific alpha-synuclein misexpression experiments (some virtually identical to those performed by Feany and Bender), also using the Gal4/UAS system, only to find that some of the PD phenotypes described by

Feany and Bender did not always occur.[42] For instance, DA neuron-specific expression of alpha synuclein (via the dopa decarboxylase driver D*dcGal4*) did not result in loss of DA neurons.[42] Furthermore, behavioral tests (specifically geotaxis experiments) failed to show significantly diminished climbing ability in flies with pan-neuronal expression of alpha-synuclein.[42] Thus, Pesah et. al. warned that

> the results of misexpression of a-Synuclein in different tissues in Drosophila should be interpreted with caution and that previously observed phenotypes may not be fully penetrant. In addition, a multiassay approach to analyze dopaminergic cell loss in DMCs is strongly recommended.[42]

Before I began stress-testing the alpha-synuclein transgenic flies, several lines of flies with known genotypes and key phenotypic characteristics had been already established and expanded at Dr. Bing Zhang's lab. Three different Gal4 pattern lines (*C155Gal4*, *D42Gal4*, and *M1BGal4*) of *Drosophila* had been mated, or were available for crossing (i.e. breeding), with four target lines of flies: *CS*, *UAS-Syn* (with its locus on the second chromosome), *UAS-Syn*^{A30P} (also located on the second chromosome), and *UAS*-Syn^{A53T} (X chromosome). "*CS*" stands for "Canton-S," which is a commonly used and well-established wild-type line of *Drosophila*, often alternatively represented by a "+"; a completely wild-type fly would therefore be "*CS* x *CS*" or "+/+." The UAS designation preceding each of the target lines (with the exception of the wild-type line) shows that they contain an upstream activation sequence to which Gal4 can bind and cause a downstream gene or multiple genes to be expressed.[36] *C155Gal4* is "an enhancer-trap insertion into the *elav* locus on the X chromosome" that causes the Gal4 driver to be present in all post-mitotic neurons.[43] Both the *C155Gal4* locus and that of *UAS*-Syn^{A53T} are on the X chromosome. Because only one of these two loci would be retained with certainty in the F1 generation (in a cross between these two pattern and target lines), a non-X-chromosomal driver was necessary; hence, a pattern line with a variant of the *C155Gal4* driver, which can be simply

called *elavGal4* (also a pan-neuronal driver, like *C155Gal4*), was used for the target line *UAS-Syn*^{A53T}. Adult flies with the *D42Gal4* enhancer trap (in the third chromosome) express Gal4 only in motor neurons (also called "motoneurons").[44]

The *M1BGal4* genotype is glial cell-specific and pertains to a locus on the fly's third chromosome.[45] Glial cells (also called "glia" or "neuroglia"), aside from neurons, are the second major cell type in the nervous system. In fact, in vertebrates, at least 50% of the cell mass in the nervous system is due to glial cells, which can "outnumber neurons by as much as 10 to 1."[40] Glial cells do not conduct nerve impulses, but instead support and help control the growth, maturation, and death of neurons; in other words, their function is to support and maintain nerve cells.[40, 46] Glial cells may play a direct role in PD; like neurons, they also form alpha-synuclein inclusions.[8] Additionally, it is possible that in PD, protein factors produced by glial cells may be insufficient or dysfunctional, leading to neuronal death (e.g. the loss of DA neurons in PD).[1]

As a short aside for readers who have not worked with *Drosophila*, sometimes specific genotypes require "virgin" females, which have not mated and thus do not carry eggs fertilized by newly eclosed males from the same, original food vials. For such crosses, fertilized eggs of the correct genotype are ensured (with a reasonably high level of confidence) by selection and separation of females shortly after eclosure – very young flies with incompletely unfurled wings and little or no pigmentation on their abdomens. The sequestered virgins are then placed in separate empty food vials and are sometimes monitored further (in case they mated unexpectedly). A useful tip for collecting virgin females: *Drosophila*, like many other organisms, follow circadian rhythms; eclosure generally occurs in the mornings. (If lights were turned on around 9:30 A.M., a significant number of adult flies would finish eclosing around 10:00 A.M. Typically, the number of eclosures would peak again at about 1:00 P.M.).

Since *UAS-Syn*^{A53T} is located on the X chromosome, for all F1 generations containing this locus, virgin females were crossed with young males from the driver lines. On the other hand, for

lines without *UAS-Syn*^{A53T}, virgin females from the driver lines were crossed with males from the target lines to ensure the right genotypes in the F1 generation. As mentioned earlier, flies with *C155Gal4* (also on X chromosome, thus requiring virgin females for crosses) mate at an earlier age than flies from the other lines, so virgin females from this line were inspected visually twice before sequestration. Since virgin females require active and careful selection, if virgin females were needed from a specific parental line, that line was expanded heavily to maximize the number of available virgins for crosses.

An example - the "correct" cross for *C155Gal4* x *UAS-Syn* (the "-" represents the lack of another X chromosome/presence of a Y chromosome in male flies):

PARENTAL (P) GENERATION

Female: *C155Gal4*/*C155Gal4* ; +/+; +/+ x

Male: +/-; *UAS-Syn*; +/+

-> F1 GENERATION

Females: *C155Gal4*/+; *UAS-Syn*/+; +/+

Males: *C155Gal4*/-; *UAS-Syn*/+; +/+

The "incorrect" version of this cross:

PARENTAL GENERATION

Female: +/+; *UAS-Syn*; +/+

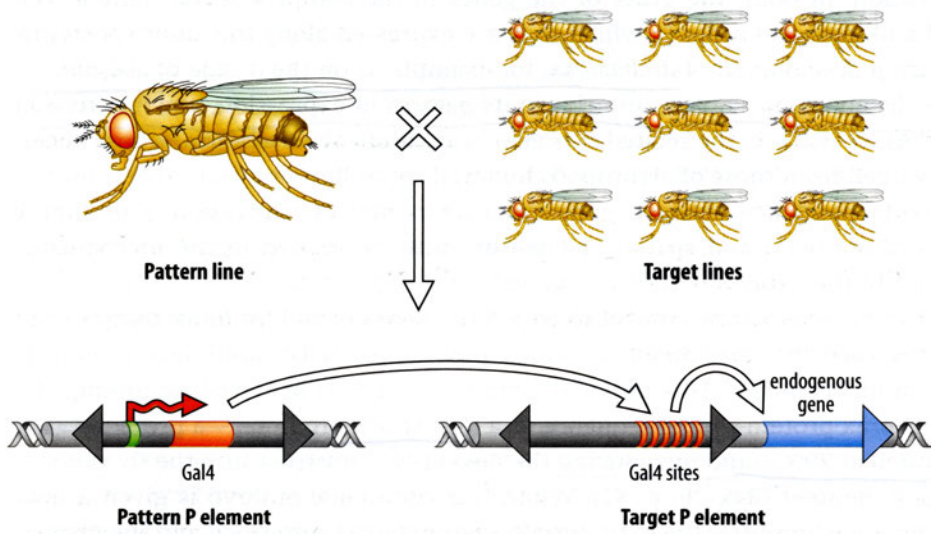
Male: *C155Gal4*/-; +/+; +/+

-> F1 GENERATION

Females: *C155Gal4*/+; *UAS-Syn*/+; +/+

Males: +/-; *UAS-Syn*/+; +/+

(Here, only the F1 females express *Syn*; the males do not).



Wolpert, L. et. al. *Principles of Development.* 3rd Ed. p. 82.

Figure 9. Targeted gene expression using a Gal4 driver. In the pattern P element, the gene for Gal4 protein (thick orange band in figure) is activated by regulatory sequences (green band) upstream of it. This Gal4 expression is specific to certain tissues in flies of the pattern line (e.g. glia only for *M1BGal4*). When crossed with flies carrying UAS (upstream activation sequence) elements for Gal4 (thin orange bands), sites where Gal4 can bind, a target gene (labeled "endogenous gene" in image; e.g. *Syn*) located downstream can be expressed.

In this way, flies of the F1 generation (of genotypes listed in Figure 10) were derived. Flies with "*CS*" in their genotype are negative controls that can provide baseline values in behavioral testing (e.g. results for a Gal4 pattern line crossed with *CS* can be validly compared with that Gal4 line crossed with *UAS-Syn*, *UAS-Syn*^{A30P}, and *UAS*-Syn^{A53T}, as opposed to just *CS* x *CS*, which does not account for the possible effects of introducing a Gal4 driver itself into the fly).

Like humans, fruit flies have diploid somatic cells. Virgin female flies of the *C155Gal4* line, for example, are homozygous for wild-type alleles on their third chromosomes (i.e. their extended genotype can be written as *C155Gal4* / *C155Gal4*; +/+; **+/+**; +/+). The third chromosomes of flies in the *M1BGal4* stock, however, are *M1BGal4* / *MKRS*. The balancer chromosome *MKRS* is used to prevent crossing over, or unpredictable exchange of genetic

information, in the "proximal regions of both arms of chromosome 3."[47] For example, we used *MKRS* in the parental/stock *M1BGal4* line, maintaining the integrity of the Gal4 gene. Yet successive mating between flies in the driver lines does not result in *MKRS* overwhelming the Gal4 genes because the balancer is homozygous lethal.[47] We screened F1 flies (e.g. for *M1BGal4* x *CS*) from the crosses between the pattern lines and the target lines before behavioral testing to ensure that the balancer was not present. Some of the F1 progeny did not have the correct genotype, which was manifested by less eye pigmentation (resulting in white or orange eyes) and/or shorter hairs (about a quarter of the normal length) on the dorsal side of their thoraxes, resembling stubble on a man's chin. Consequently, only red-eyed, non-stubble flies were kept.

				Sample Size (n values)			
				# of Starvation Vials		# of Paraquat Vials	
Pattern Lines	**Tissue/Location of Expression**		**Crosses (F1 Flies)**	**Males**	**Females**	**Males**	**Females**
			CS x CS	8	9	16	12
			CS x UAS-Syn	-	-	-	-
			CS x UAS-Syn^A30P	-	-	-	-
CS	N/A (Wildtype Control)	->	CS x UAS-Syn^A53T	-	-	-	-
			C155Gal4 x CS	13	13	10	9
			C155Gal4 x UAS-Syn	10	29	14	12
			C155Gal4 x UAS-Syn^A30P	30	22	9	8
C155Gal4	Neurons	->	C155Gal4 x UAS-Syn^A53T	18	19	24	21
			D42Gal4 x CS	17	16	11	10
			D42Gal4 x UAS-Syn	19	18	19	11
			D42Gal4 x UAS-Syn^A30P	8	11	17	13
D42Gal4	Motoneurons	->	D42Gal4 x UAS-Syn^A53T	17	15	13	18
			M1BGal4 x CS	6	4	10	9
			M1BGal4 x UAS-Syn	9	11	15	12
			M1BGal4 x UAS-Syn^A30P	14	13	18	16
M1BGal4	Glia	->	M1BGal4 x UAS-Syn^A53T	10	5	13	13

Figure 10. The crosses of interest derived from three tissue-specific Gal4 pattern lines and four target lines. *CS* (wild-type) flies serve as controls.

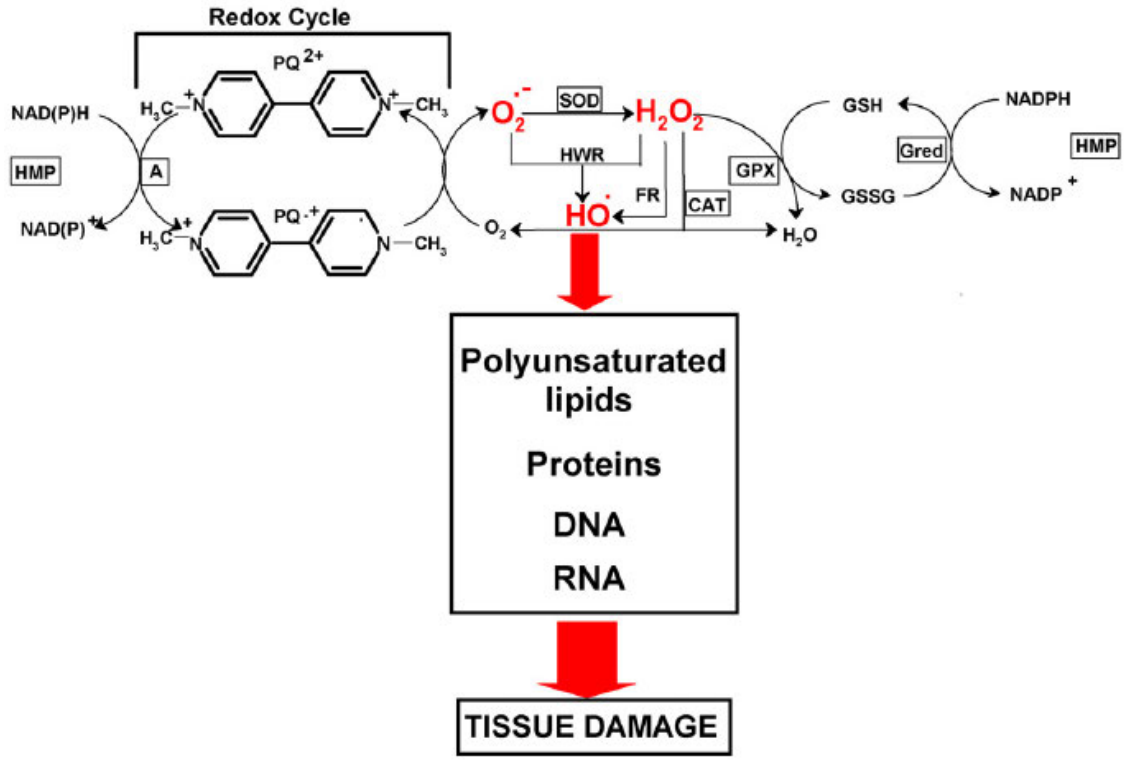
Behavioral Testing

The behavioral testing was accomplished in a separate room, away from the fly stockroom (at 23.0 ± 0.2 degrees Celsius) and any windows. To measure their sensitivity to environmental factors, young (one- to three-day-old) F1 transgenic flies were subjected to two forms of stress: "wet" starvation and paraquat exposure. Wet starvation is an assay that involves "placing groups of flies in a vial or bottle giving them access to water but not food, and then monitoring times until death."[48] Since -at a fundamental level- both environmental and genetic factors affect behavior, we expected that different genotypes of alpha-synuclein transgenic flies would have different responses, in terms of longevity, to metabolic stress induced by starvation. Because the conditions for the starvation testing are very similar to those in the paraquat exposure testing, the results from both sets of experiments can also be compared.

Measuring starvation resistance with wet starvation testing is valid, because though *Drosophila* larvae are "facultative carnivores," adult flies "do not...derive a measurable survival benefit from cannibalism."[48] The flies for this assay were collected in the morning and placed in empty, transparent plastic vials in groups of roughly nine or ten flies. After six hours, drops of double-distilled water were added to the corks of the empty vials (with disposable pipettes), until the lower third of the corks (i.e. the side facing the flies) were fully moistened. The flies were transferred from the empty food vials to the wet starvation vials by swift dumping and concussing (i.e. the empty food vials were quickly inverted over the wet starvation vials, and the wet starvation vials were tapped against a lab table until the flies were all securely transferred). The transfers were done with conscious flies; no CO_2 anesthesia or ice was used. The time was then recorded, and thereafter, at that time of day (i.e. every 24 hours), I recorded of the number of dead flies in each vial and replenished the available water by adding five drops to each of the corks, if they were not visibly moist (saturating the corks led to mold growth).

One of the major theories about PD mechanisms is that oxidative stress leads to cell

death.[1] An oxidative agent that is associated with PD is paraquat (also known as "methyl viologen dichloride hydrate"; chemical formula: $C_{12}H_{14}C_{l2}N_2$; FW: 257.17 Da; melting point: greater than 300 °C), commonly used as an herbicide.[7] There are several possible mechanisms. Paraquat seems to damage the human central nervous system via reactive oxygen species (such as $O_2^{\cdot -}$, an unstable radical; see Figure 11 for examples) that hinder mitochondrial function and may lead to decreased dopamine synthesis.[7] Furthermore, paraquat seems to cause the production of damaging reactive nitrogen species and cause increased levels of alpha-synuclein synthesis and aggregation, perhaps resulting in Lewy body formation; paraquat-stimulated "long-lasting dopamine overflow" can lead to DA neuron loss.[7] Mammalian DA neurons may be especially sensitive to paraquat due to the presence of a special enzyme that transports a protective compound (GSH, or the reduced form of glutathione) out of the glia associated with these cells.[7]



Dinis-Oliveira, R.J., et. al., 2006. Review: Paraquat exposure as an etiological factor of Parkinson's disease. NeuroToxicology

27, 1110–1122.

Figure 11. The Structure of Paraquat and "Mechanism of Paraquat Toxicity." What the abbreviations in the above diagram represent: "A, cellular diaphorases; SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase; Gred, glutathione reductase; PQ2+, paraquat; PQ+, paraquat cation radical; HMP, hexose monophosphate pathway; FR, Fenton reaction; HWR, Haber-Weiss reaction."[7] In this schematic, paraquat and its radical form can be found under the section of the diagram labeled "Redox Cycle"); two chloride ions (not in figure) are also present, neutralizing the net charge of paraquat. The three species labeled in red font (superoxide radical [$O_2^{\cdot -}$], hydroxyl radical [$HO^{\cdot}$], and hydrogen peroxide [H_2O_2]) are reactive oxygen species that form a perpetuating "redox cycle" and can oxidize lipids, proteins, and nucleic acids, leading to membrane and tissue damage.[7] Nitric oxide ($^{\cdot}NO$) and peryoxynitrite anion ($ONOO^-$) are also formed by paraquat exposure.

By subjecting various genotypes of alpha-synuclein transgenic fruit flies to paraquat exposure (under similar controlled conditions), it is possible to quantify their relative sensitivity to the oxidative agent. As in the wet starvation testing, we expected paraquat to have different effects on the longevities of different combinations of pattern and target lines of flies. For paraquat exposure testing, I prepared a 20 mM paraquat (Aldrich Chemistry, 98%; CAS No.: 1910-42-5) in 5% sucrose solution (sucrose: Sigma-Aldrich; the solution was prepared using 0.25715 gram of paraquat, 2.5 grams of sucrose, and enough double-distilled H_2O to make 50.0 mL of solution; in general, 5.143 mg of paraquat is needed per 1.0 mL of solution) and applied 5 drops, with a disposable plastic pipette, to an empty food vial with a small filter paper circle lining the bottom of the tube. The filter paper linings were cut from larger filter paper circles (Whatman, Qualitative Medium). As with the wet starvation testing, the flies were placed and starved in empty food vials for 6 hours before being transferred to the test/paraquat vials. The hungry and thirsty flies would then ingest the paraquat-sucrose solution, and as in starvation testing, I would record the number of deceased flies in each paraquat testing vial.

Results

Control Flies

CS (or Canton-S, as mentioned earlier) and OR are strains of *Drosophila* that are commonly used in biological studies as designated wild-type flies.[49] For behavioral testing (i.e. the wet starvation and paraquat exposure experiments), both *CS* flies and *CS* flies expressing tissue-specific Gal4 drivers were used (e.g. *C155Gal4* x *CS*); the *CS* flies with Gal4 protein served as additional controls and, due to greater genetic similarity with the F1 alpha-synuclein transgenic flies, are more appropriate for comparison than purely *CS* flies. This idea is reinforced by the wet starvation data (displayed in Figures 12a-f). All female *CS* flies subjected to wet starvation died earlier than their counterparts with the Gal4 driver. This trend was even more noticeable (and applied to male flies of the respective genotypes as well) in the data (displayed in Figures 13a-f) from the paraquat exposure experiments, "suggesting that *CS* flies may have accumulated modifiers that reduce their stress handling capabilities."[49] Furthermore, the results show the importance of incorporating genetically-compatible control flies in future studies of fly aging, survival, or any sort of time-dependent response to stress. Thus, instead of *CS* x *CS* flies, the *CS* flies with tissue-specific Gal4 driver will serve as the controls in the analysis of F1 fly results.

Males vs. Females

On the whole, F1 females were more resistant to starvation than the males. This was true for both starvation and paraquat experiments and for all genotypes, including the control flies. For example, due to wet starvation, all of the F1 *CS* male flies with Gal4 overexpressed in their

neurons (i.e. *C155Gal4* x *CS*) were dead after 120 hours (or 5 days), as opposed to 168 hours (or 7 days) for females of the same genotype. (In contrast, under typical laboratory conditions – normoxia, with flies transferred to fresh food vials every 2-3 days, and so on – CS flies have a lifespan of about 30 days and can live to more than twice that long).[50] Flies with Gal4 driver in their motorneurons survived slightly longer (192 hours for males and 216 hours for females). Of the F1 Gal4 control flies, the greatest lifespan difference (for wet starvation testing) between males and females occurred in the *CS*/glial-cell specific Gal4 flies. However, this can be accounted for and may have been due to a suboptimal sample size for the females of this genotype and the fact that one female survived for more than 264 hours. With the exception of control flies with glial Gal4, in the paraquat exposure testing, the times required for all F1 males of the control genotypes (*CS* with driver) to die versus F1 females were less different (within 24 hours) than in the wet starvation testing.

Wet Starvation

1. Glial Overexpression

Compared to the *M1BGal4* x *CS* controls, *M1BGal4* x UAS-*Syn* F1 males (flies with glial cell-specific overexpression of alpha-synuclein) all experienced early death, suggesting that the transgenes enhanced starvation stress and thus accelerated fly death. Of the males with glial overexpression of trangene, flies with wild-type alpha-synuclein had the shortest population half-life (time required for 50% of the flies to die, or $t_{1/2}$) of 60 hours (or five days). The $t_{1/2}$ for males with Syn^{A53T} in glia was about 80 hours. For Syn^{A30P}, $t_{1/2}$ was about 95 hours (the highest $t_{1/2}$ of the three forms of transgene).

In females with glial expression of the trangenes, only *Syn* and Syn^{A30P} (but not Syn^{A53T}) accelerated/enhanced fly death; $t_{1/2}$ values were significantly reduced to approximately 110 and

100 hours, respectively (with associated two-tailed p values of 0.0342 and 0.0018), from about 153 hours for the associated control flies.[51]

2. Motorneuron Overexpression

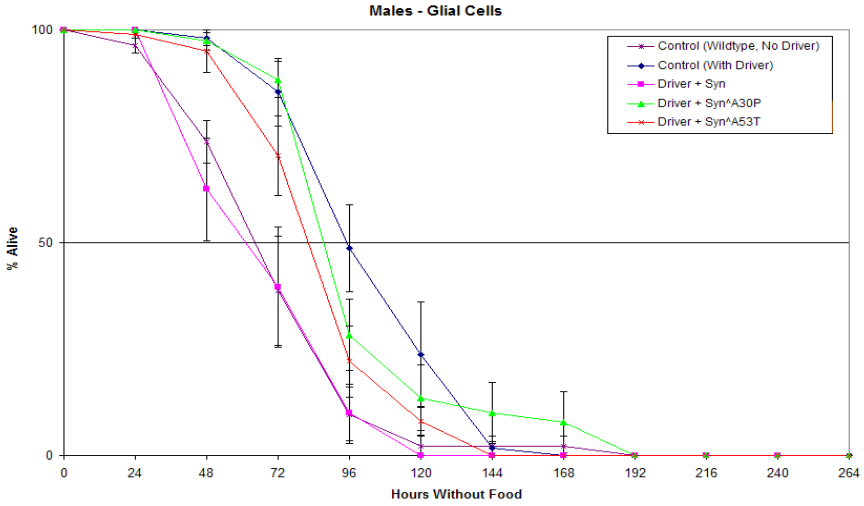
The results did not reveal any consistent, significant effects due to the transgenes on the lifespans of both male and female flies with *D42Gal4* in their genotypes. Female *Syn* flies had the highest $t_{1/2}$ (almost 110 hours, compared to the next highest value, 95 hours for Syn^{A53T} females), though males with this alpha-synuclein transgene had a $t_{1/2}$ nearly identical to that of the associated control males (of the genotype *D42Gal4* x *CS* and with a $t_{1/2}$ of about 68 hours). The lowest $t_{1/2}$ belonged to Syn^{A30P} males ($t_{1/2}$ = 51 hours), but the females with this genotype/transgene had a $t_{1/2}$ similar to the female control flies (after accounting for uncertainty).

3. Pan-neuronal Overexpression

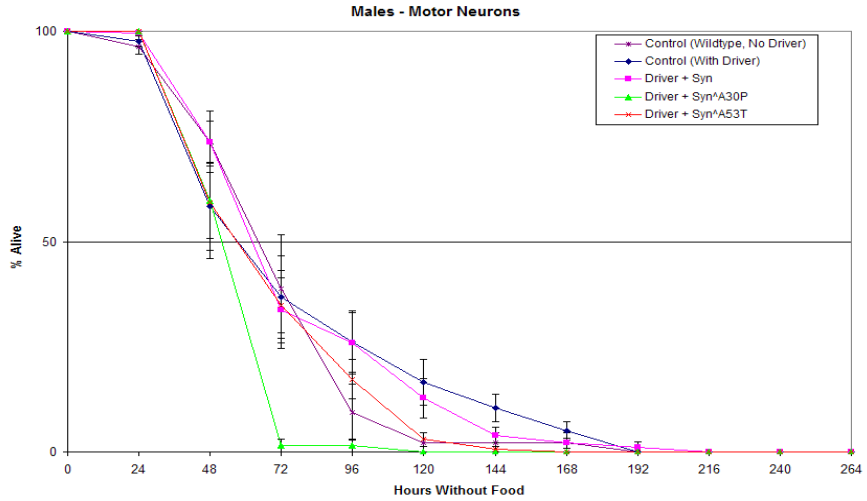
Overall, the results did not reveal any significant effects due to the transgenes on the lifespan of both male and female flies with *C155Gal4* (or *ElavGal4*) in their genotypes. However, both Syn^{A30P} and Syn^{A53T} reduced $t_{1/2}$ for females from about 115 hours (the $t_{1/2}$ for female control flies and for *Syn*) to less than 95 hours (p = 0.0037 and 0.0005, respectively).[51]

In general, the F1 *M1BGal4* crosses (i.e. flies with the alpha-synuclein transgenes) seemed to have the largest $t_{1/2}$ values (when comparing with female flies), followed by the *C155Gal4* crosses. The *D42Gal4* crosses were the least resistant to wet starvation. Though the F1 males in the starvation testing typically did not survive as long as the F1 females, the population half-lives for the males fell roughly in the same order (from longest to shortest $t_{1/2}$) as that for the females: *M1BGal4*, *C155Gal4*, and *D42Gal4*, respectively.

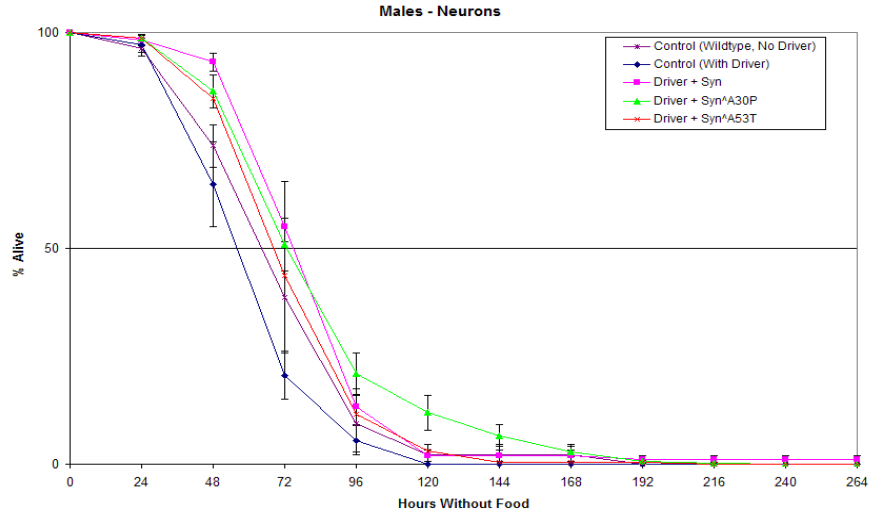
(12a)


Males - Glial Cells
Control (Wildtype, No Driver)
Control (With Driver)
Driver + Syn
Driver + Syn^A30P
Driver + Syn^A53T
% Alive
100
50
0
0
24
48
72
96
120
144
168
192
216
240
264
Hours Without Food

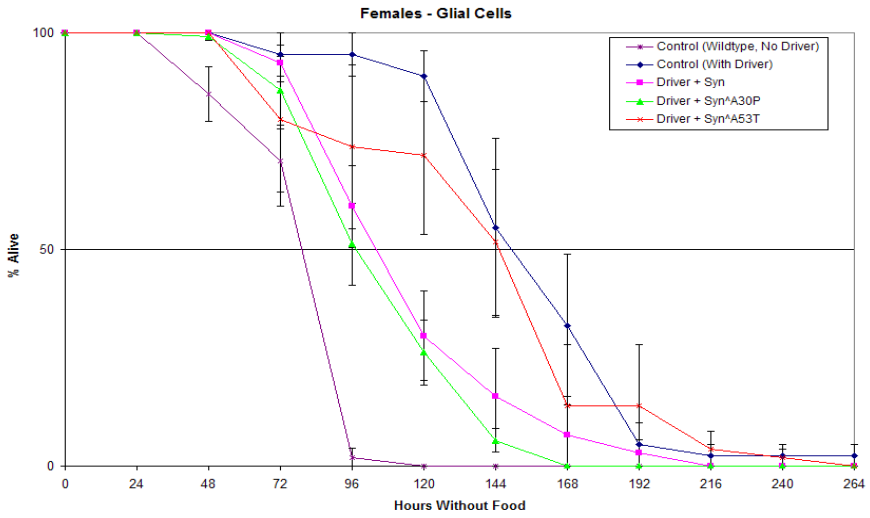
(12b)


Males - Motor Neurons
Control (Wildtype, No Driver)
Control (With Driver)
Driver + Syn
Driver + Syn^A30P
Driver + Syn^A53T
% Alive
100
50
0
0
24
48
72
96
120
144
168
192
216
240
264
Hours Without Food

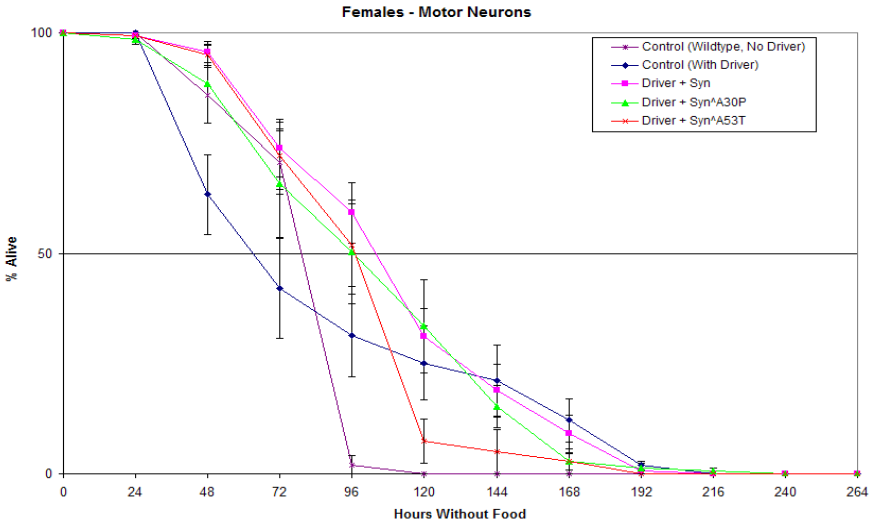
(12c)


Males - Neurons
Control (Wildtype, No Driver)
Control (With Driver)
Driver + Syn
Driver + Syn^A30P
Driver + Syn^A53T
% Alive
100
50
0
0
24
48
72
96
120
144
168
192
216
240
264
Hours Without Food

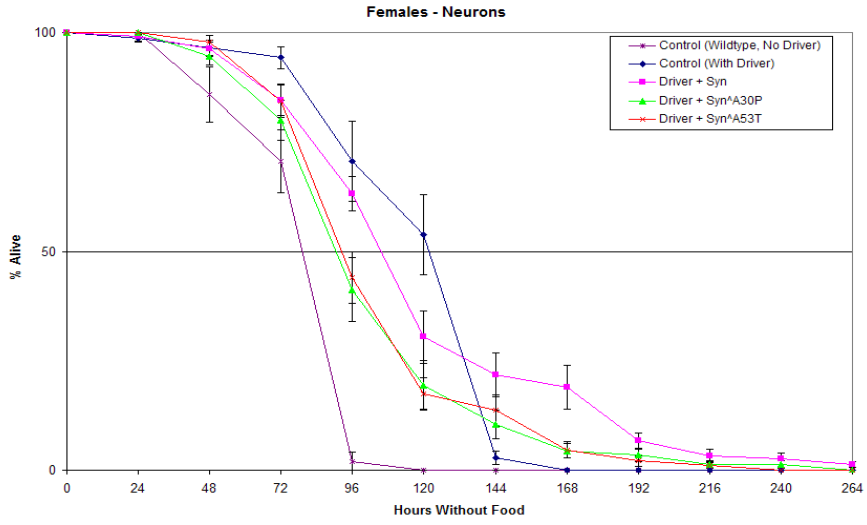
(12d)


Females - Glial Cells
Control (Wildtype, No Driver)
Control (With Driver)
Driver + Syn
Driver + Syn^A30P
Driver + Syn^A53T
% Alive
Hours Without Food
0
50
100
0
24
48
72
96
120
144
168
192
216
240
264

(12e)


Females - Motor Neurons
Control (Wildtype, No Driver)
Control (With Driver)
Driver + Syn
Driver + Syn^A30P
Driver + Syn^A53T
% Alive
Hours Without Food
0
50
100
0
24
48
72
96
120
144
168
192
216
240
264

(12f)


Females - Neurons
Control (Wildtype, No Driver)
Control (With Driver)
Driver + Syn
Driver + Syn^A30P
Driver + Syn^A53T
% Alive
Hours Without Food
0
50
100
0
24
48
72
96
120
144
168
192
216
240
264

Figures 12a-12f. Wet Starvation Data – M1BGal4 (Glial Cell-Specific Driver) F1 Flies Seem to Have Longest Population Half-Life

These graphs show the percent of flies alive versus the time they were subjected to testing (i.e. with water but not food). The uncertainty assigned for each data point (for each vial of flies), represented by the error bars in each chart, is the calculated standard error, which is equivalent to the standard deviation divided by the square root of the sample size (i.e. number of vials). The glial cell-specific Gal4 driver used is *M1BGal4;* for instance, in Figure 12a, the genotype of the flies represented as "Control (With Driver)" is *M1BGal4 x CS*. Likewise, the motor neuron-specific driver is *D42Gal4*, and the pan-neuronal driver used for the three alpha-synuclein target lines and one of the control populations is *C155Gal4* (and *ElavGal4, functionally equivalent to C155Gal4,* for the UAS-Syn^{A53T} line). Figure 10 contains more details about genotypes and sample sizes.

Paraquat Testing

1. Glial Overexpression

Overall, the results did not reveal any significant effects due to the transgenes on the lifespan of both male and female flies with *M1BGal4* in their genotypes. Yet the $t_{1/2}$ for *Syn* males was 64 hours, very noticeably reduced ($p = 0.0007$), when compared with 117 hours for the associated male control flies (*M1BGal4* x *CS*).[51] There was also a large disparity between the $t_{1/2}$ differences between males and females of the same genotype. *Syn*^{A30P} males had a $t_{1/2}$ of about 119 hours, compared with a $t_{1/2}$ value of 129 hours for the *Syn*^{A30P} females (a difference of 10 hours). On the other hand, the $t_{1/2}$ for *Syn*^{A53T} males and females were approximately 26 (like that for *Syn* males, much lower than the control flies) and 144 hours, respectively (a much larger difference of 118 hours).

2. Motorneuron Overexpression

The results did not reveal any overall significant effects due to the transgenes on the lifespan of both male and female flies with *D42Gal4* in their genotypes. However, as with male flies overexpressing wild-type human alpha-synuclein in their glial cells, males with *Syn* expressed in motorneurons had a noticeably lower $t_{1/2}$ value (75 hours; $p = 0.0062$) when

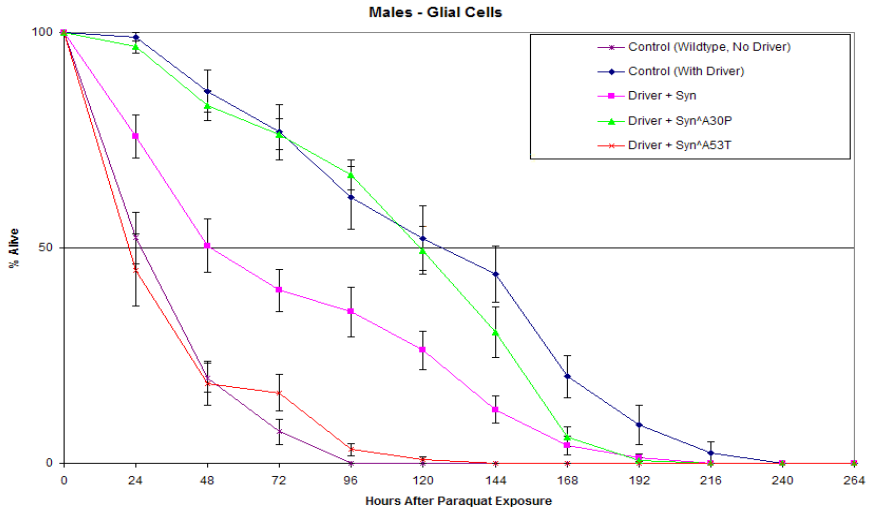
compared to the appropriate control flies ($t_{1/2}$ = 108 hours).[51]

3. Pan-neuronal Overexpression

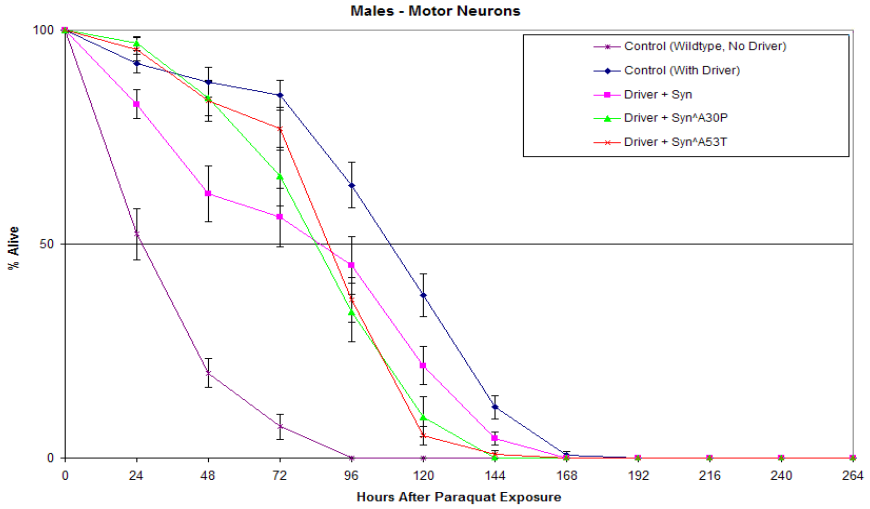
In both males and females, all three transgenes (*Syn*, *Syn*^{A30P}, and *Syn*^{A53T}) enhanced accelerated death (i.e. led to significantly reduced $t_{1/2}$ values) due to paraquat exposure, an effect noticeable even after considering uncertainty; comparing the $t_{1/2}$ values of the control and transgenic flies produced "a difference considered extremely statistically significant" (two-tailed $p = 0.0003$ for *Syn*^{A30P} females; two-tailed $p < 0.0001$ for other groups of transgenic flies, male or female).[51] The male control flies had a $t_{1/2}$ of about 141 hours, whereas female controls had a $t_{1/2}$ of about 153 hours. The *Syn* transgene was the most effective in reducing lifespan ($t_{1/2}$ = 50 hours for males, 86 hours for females). This was followed by *Syn*^{A30P} (with a $t_{1/2}$ of 56 hours for males and about 102 for females). Male *Syn*^{A53T} flies had a $t_{1/2}$ of about 67 hours, and for females of the same genotype, $t_{1/2}$ was about 115 hours.

In general, as in the wet starvation testing, female flies in the paraquat testing had longer lifespans than their male counterparts (as mentioned earlier), as evidenced by the distributions of the flies' $t_{1/2}$ values for each distinct genotype. Flies of the genotype *M1BGal4* x *UAS-Syn*^{A53T} genotype have a survival curve that closely resemble that of the *CS* x *CS* genotype. Yet even in this case, the curve for *CS* x *CS* flies, at 96 hours (or four days), reaches a value of 0% flies alive faster than the one for glial cell-specific expression of alpha-synuclein. It is reassuring to note that the *CS* x *CS* curve for both males and females resembles the curve for the control flies of the paraquat exposure experiments done by Meulener et. al. on DJ-1 flies; like the flies in my experiments, the control flies for their experiments were also exposed to 20 mM paraquat, resulting in a curve with a population half-life of about 24 hours (1 day).[19]

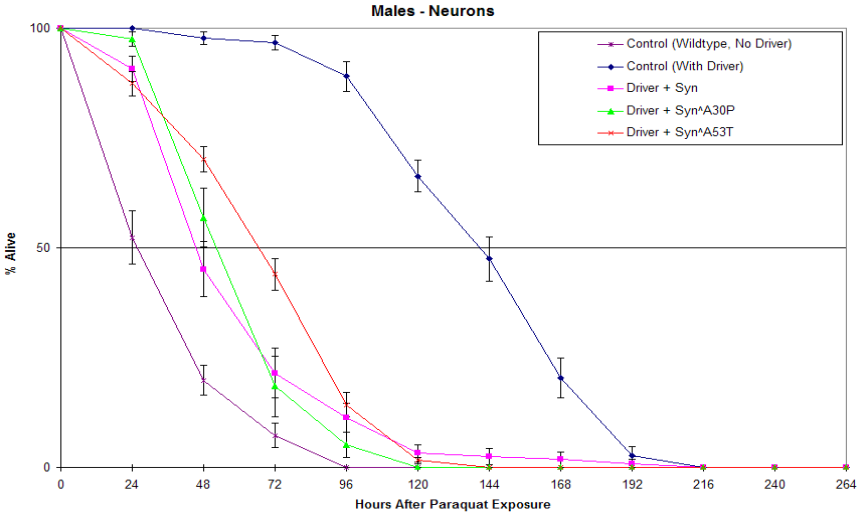
(13a)


Males - Glial Cells
Control (Wildtype, No Driver)
Control (With Driver)
Driver + Syn
Driver + Syn^A30P
Driver + Syn^A53T
% Alive
100
50
0
0
24
48
72
96
120
144
168
192
216
240
264
Hours After Paraquat Exposure

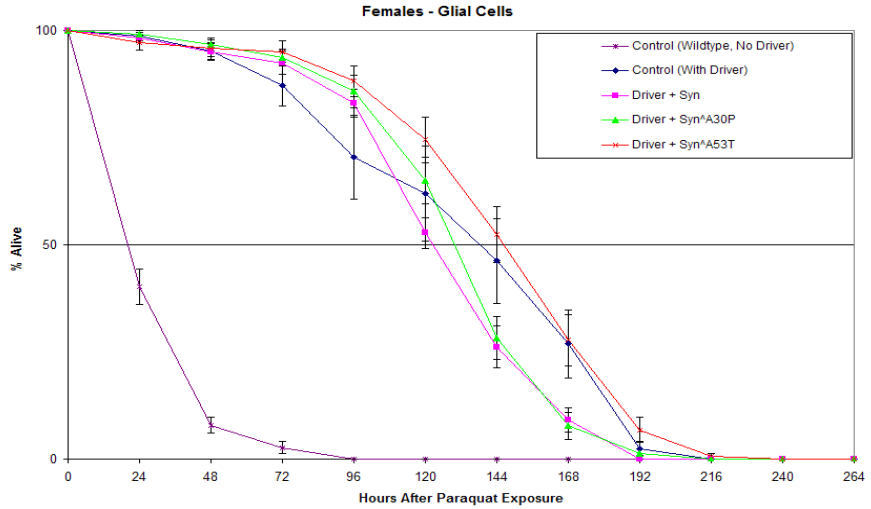
(13b)


Males - Motor Neurons
Control (Wildtype, No Driver)
Control (With Driver)
Driver + Syn
Driver + Syn^A30P
Driver + Syn^A53T
% Alive
100
50
0
0
24
48
72
96
120
144
168
192
216
240
264
Hours After Paraquat Exposure

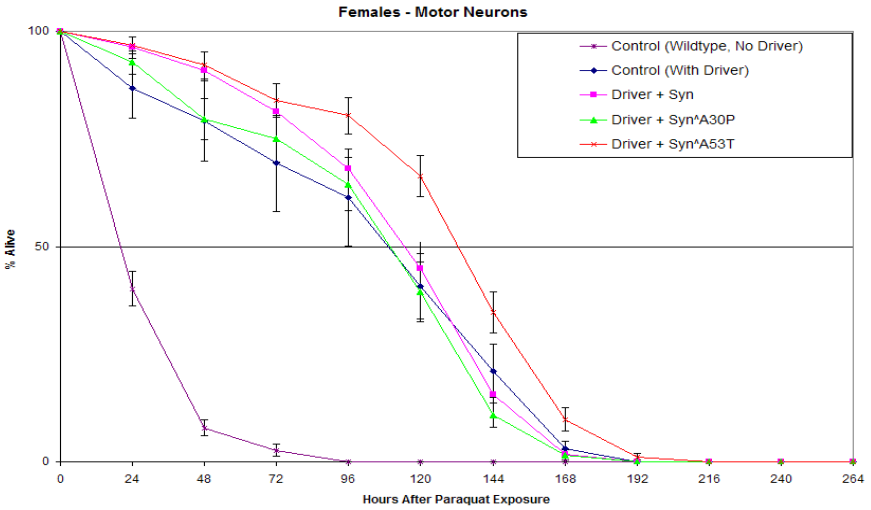
(13c)


Males - Neurons
Control (Wildtype, No Driver)
Control (With Driver)
Driver + Syn
Driver + Syn^A30P
Driver + Syn^A53T
% Alive
100
50
0
0
24
48
72
96
120
144
168
192
216
240
264
Hours After Paraquat Exposure

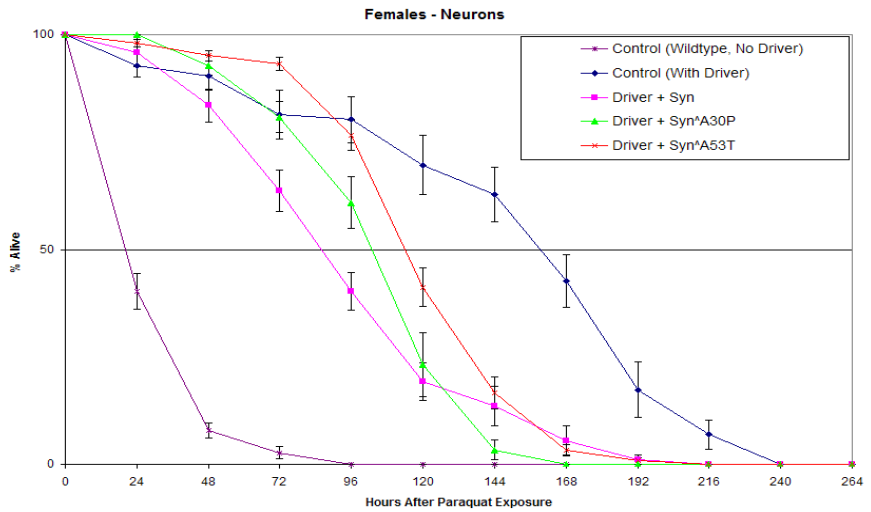
(13d)


Females - Glial Cells
Control (Wildtype, No Driver)
Control (With Driver)
Driver + Syn
Driver + Syn^A30P
Driver + Syn^A53T
% Alive
100
50
0
0
24
48
72
96
120
144
168
192
216
240
264
Hours After Paraquat Exposure

(13e)


Females - Motor Neurons
Control (Wildtype, No Driver)
Control (With Driver)
Driver + Syn
Driver + Syn^A30P
Driver + Syn^A53T
% Alive
100
50
0
0
24
48
72
96
120
144
168
192
216
240
264
Hours After Paraquat Exposure

(13f)


Females - Neurons
Control (Wildtype, No Driver)
Control (With Driver)
Driver + Syn
Driver + Syn^A30P
Driver + Syn^A53T
% Alive
100
50
0
0
24
48
72
96
120
144
168
192
216
240
264
Hours After Paraquat Exposure

Figures 13a-13f. Paraquat Exposure Data – Except for Glial Cells, *Syn*^{A53T} Have Highest $t_{1/2}$ of Transgenic Flies Since the set of genotypes subjected to paraquat exposure is the same as that for the starvation testing, the legends and representations for these graphs are identical to those of the graphs of the data from the wet starvation testing (Figures 12a-f). With the exception of Figure 13a, the paraquat testing graphs quite distinctly show that wild-type control flies (*CS* x *CS*) have the shortest lifespan in these experiments, with the last fly dying after a maximum of 96 hours (4 days) in both male and female populations. Ordering, from shortest to longest time required, the flies expressing alpha-synuclein (exclusively in glial cells, motoneurons, or all post-mitotic neurons) by population half-lives generally gives wild-type human alpha-synuclein, alpha-synuclein with the A30P mutation, and then A53T alpha-synuclein, which does not correctly model the human phenotypes; humans with wild-type alpha-synuclein are not expected to have shortened lifespans, which is what occurs in *Drosophila*.

Discussion and Conclusions

Data collected from starvation testing show the importance and influence of food availability on the longevity of *Drosophila melanogaster*. For many of the flies (e.g. *CS* x *CS* females), starvation-induced stress was nearly as lethal as paraquat toxicity, with differences of last-fly deaths (i.e. time required for population to reach 0% alive) on the order of a day or two between the two tests (when comparing identical genotypes). On the whole, flies with glial cell-specific Gal4 expression had longer population half-lives than those with motorneuronal or pan-neuronal driver expression; however, confirmation of this finding (through further experiments) will be necessary. The uncertainty of the starvation data can be reduced by repeating these tests, which will reduce the impact of random, minor fluctuations in environmental conditions on the averaged results.

The data generated via paraquat exposure testing with transgenic flies expressing wild-type human alpha-synuclein (and two of its three known mutant forms: A30P or A53T) do not truly correspond with what is known about the PD phenotype (in terms of lifespan), specifically that of alpha-synuclein-related familial PD. However, after comparing the time required for all flies (in each genotypically unique population) to die, F1 flies with pan-neuronal Gal4 drivers

(*C155Gal4* and *ElavGal4*) and alpha-synuclein, compared with lines expressing alpha-synuclein in glial cells or motor neurons, seem to most closely follow what is expected of a model organism for PD. In other words, the reasonable expectation that flies expressing wild-type alpha-synuclein in their neurons outliving flies with mutated alpha-synuclein (i.e. A30P or A53T) was fulfilled. To potentially support and amplify the trends identified from the paraquat exposure data, more testing (perhaps with other genotypic combinations, such as ones with DA-neuron-specific *DdcGal4* as a pattern/driver line) and experiments with different oxidants, such as rotenone and hydrogen peroxide, will be necessary on the genotypes that have already been tested.[19, 23] Hydrogen peroxide is a good chemical agent for oxidative stress experiments, because it is readily available (e.g. Fisher Scientific H_2O_2 – 30%; b.p. 108 degrees C; stabilized with sodium stannate; CAS 7722-84-1 and 7732-18-5). Stabilized hydrogen peroxide is advised for prolonged experiments, since hydrogen peroxide readily decomposes into water and oxygen. I began a few vials for hydrogen peroxide testing but did not complete enough of them to present the data without statistical artifacts. The sample sizes for these flies are just too low for the data to be meaningful.

Overall, my research is a very preliminary look at the effect of stress on transgenic flies with human alpha-synuclein. Other kinds of testing/experiments would be helpful in exploring the effects of introducing the PD-associated protein. Observing the number of DA neurons in the combinations of fly genotypes that I experimented on and comparing them with that of wild-type flies would be a meaningful quantitative assessment of alpha-synuclein-related damage, especially if the values were recorded at various time points (e.g. dissecting living flies at the population half-life).[9, 23] Tyrosine hydroxylase (TH) is the mixed function oxidase that catalyzes L-dopa formation from tyrosine (see Figure 1) and is found in DA neurons, which thus can be identified by staining for TH.[52] In fact, I learned how to dissect *Drosophila*, extract the central nervous system (the fly CNS is composed of a brain and central nerve cord), and attempted to

stain the DA neurons of the fly CNS several times. Ironically and unfortunately, the immunohistochemical staining (involving a 1:100 dilution of rabbit anti-TH primary antibody and a 1:100 dilution of goat anti-rabbit secondary antibody, with the fluorescent tag TRITC) was successful only once -the first time I attempted it. The techniques I used for the staining seem fairly reasonable, and I made slight modifications of the in procedure, in hopes of seeing beautifully stained and emphasized DA neurons. However, since this did not happen, antibody staining is something that can be explored in the future.

Working with alpha-synuclein transgenic flies was inherently interesting and absorbing. Yet, it is important to remember that research on PD should ultimately contribute to some larger picture and greater understanding of the disease state. With the combined efforts of researchers using different approaches and biological methods, we are closer to successfully understanding, preventing, and treating neurodegenerative diseases and other ailments that have confounded humans for ages. Hopefully, further research will contribute to making PD and other neurodegenerative disorders specters of the past for future generations and provide more knowledge about our nervous systems and about ourselves.

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